

REMARKS

The specification has been amended to claim benefit of prior filed applications in accordance with 37 C.F.R. § 1.78(a)(2), to conform to current Patent Office practice requiring reference to the lettered subparts of the formal drawings submitted herewith, to correct typographical errors, to update the address of the American Type Culture Collection, to incorporate sequence identifiers pursuant to 37 C.F.R. § 1.821(d), to incorporate the Substitute Sequence Listing submitted herewith pursuant to 37 C.F.R. § 1.821(c) and renumber the pages of the claims accordingly. Additionally, the specification has been amended on page 5 to correct the location of the pentapeptide QACRG in SEQ ID NO:2. No new matter has been added.

A substitute sequence listing and substitute Figures 1A-B and 3A-C are submitted herewith to correct nucleotide and amino acid sequence errors as originally presented in the ICE-LAP 3 sequences (shown in the sequence listing as SEQ ID NOS:1 and 2). These changes do not introduce new matter because the correct sequence was inherent to the originally sequenced plasmid clone in actual possession of the Applicants before the time the captioned application was filed.

The captioned application is directed to both polynucleotides and polypeptides which are chemical compounds. The nucleotide sequence is but an inherent property of the described polynucleotides. There is a line of chemical case law where applicants have been permitted to amend the specification to correct the formula of a chemical compound after an application's filing date, provided that it had been demonstrated that one of skill in the art would have appreciated that the applicant was in possession of the compound itself at the time of filing. The rationale is that the formula is an inherent property of the compound and thus amending the specification to correct the formula is not new matter. See *In re Nathan*, 140 U.S.P.Q. 601, 604 (C.C.P.A. 1964). *Accord Kennecott Corp. v. Kyocera Int'l, Inc.*, 5 U.S.P.Q.2d 1194, 1198 (Fed. Cir. 1987), *cert. denied*, 486 U.S. 1008 (1988) ("The disclosure in a subsequent patent application of an inherent property of a product does not deprive that product of the benefit of the earlier filing date.").

In the field of biotechnology, applicants often rely on a deposited clone, where the deposit was made prior to filing, to establish possession of nucleic acids or proteins. The focus for determining whether applicants were in possession of claimed nucleic acids or proteins has been determined, at least in part, by considering whether the applicant has: (1) established that one skilled in the art in possession of the deposited clone would have been aware of both the DNA sequence and the encoded amino acid sequence, or would be able to determine these sequences without undue experimentation, (2) established that the DNA and amino acid sequences are described in a manner such that one skilled in the art could distinguish them from other sequences, and (3) resequenced a clone which is identical to that of the deposit and established a "chain of custody" for this clone. See

e.g., *Ex parte Maizel*, 27 U.S.P.Q.2d 1662, 1669-1670 (B.P.A.I. 1992).

Submitted herewith is a Declaration of Craig Rosen Under 37 CFR §1.132 (the "Rosen Declaration") which was filed in connection with the prosecution of parent application Serial Number 08/462,969, and which describes certain events involving the characterization of the nucleotide sequence of cDNA clone HE2CA82 which encodes ICE-LAP 3. The HE2CA82 cDNA clone was deposited with the American Type Culture Collection (ATCC) on August 25, 1994; i.e., prior to the filing date of parent application US Serial No. 08/334,251 (filed November 1, 1994). Exhibit A of the Rosen Declaration is a copy of the contract for ATCC Deposit No. 75875. The Examiner will note that the present specification states on page 5, lines 7 and 8, that ATCC Deposit No. 75875 contains the cDNA which encodes ICE-LAP 3.

The corrected nucleic acid and deduced amino acid sequences (shown in replacement Figures 1 and 3 submitted herewith) were determined by reanalyzing cDNA clone HE2CA82 (ATCC Accession No. 75875); i.e., the same cDNA clone from which the originally presented sequences were determined (see paragraph 4 of the Rosen Declaration). The corrected sequence information was published by the present inventors in a peer-reviewed scientific journal article (see Exhibit B of the Rosen Declaration).

The analysis needed to determine the complete and correct sequences of cDNA clone HE2CA82 were well within the skill of the ordinary artisan as of the filing date of the parent application US Serial No. 08/334,251 (filed November 1, 1994), and such analysis would not have required undue experimentation (see paragraph 5 of the Rosen Declaration).

Exhibit D of the Rosen Declaration shows the four (4) nucleotide differences between the original sequence ("PF140" in the Exhibit) and the corrected sequence ("Duan" in the Exhibit).

In summary, because Applicants have demonstrated that the corrected sequences are inherently present in the deposited material, and because Applicants have demonstrated "chain of custody" for the material originally sequenced and the resequenced material, correction of the originally presented sequence information herein is not new matter.

Statement Under 37 C.F.R. §§ 1.821(e) and (f)

The above-identified patent application is a divisional of application Serial No. 08/462,969, filed June 5, 1995. The content of the paper copy of the Substitute Sequence Listing filed herewith is identical to the sequence content of the computer readable sequence listing previously filed on March 22, 1999 in connection with application Serial No. 08/462,969.

In accordance with 37 C.F.R. § 1.821(e), please use the computer readable form filed on March 22, 1999 in connection with application Serial No. 08/462,969 as the



American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: (301)231-5520 Telex: 898-055 ATCCNORTH • FAX: 301-770-2587

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

10866 U.S. PTO
09/613508
07/10/00

To: (Name and Address of Depositor or Attorney)

Human Genome Sciences, Inc.
Attention: Craig A. Rosen, Ph.D.
9620 Medical Center Drive, Suite 300
Rockville, MD 20850

Deposited on Behalf of: Human Genome Sciences, Inc.

Identification Reference by Depositor:

ATCC Designation

DNA Plasmid:

331,608
313,903
331,607

75873
75874
75875

The deposits were accompanied by: ☐ a scientific description ☐ a proposed taxonomic description indicated above.

The deposits were received August 25, 1994 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

☒ We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested September 2, 1994. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Annette L. Bade, Head, ATCC Patent Depository
cc: Greg Ferraro

Date: September 6, 1994

ICE-LAP3, a Novel Mammalian Homologue of the *Caenorhabditis elegans* Cell Death Protein Ced-3 Is Activated during Fas- and Tumor Necrosis Factor-induced Apoptosis*

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Members of the ICE/*ced-3* gene family have been implicated as components of the cell death pathway. Based on similarities with the structural prototype interleukin-1 β -converting enzyme (ICE), family members are synthesized as proenzymes that are proteolytically processed to form active heterodimeric enzymes. In this report, we describe a novel member of this growing gene family, ICE-LAP3, which is closely related to the death effector Yama/CPP32/Apopain. Pro-ICE-LAP3 is a 35-kDa protein localized to the cytoplasm and expressed in a variety of tissues and cell lines. Overexpression of a truncated version of ICE-LAP3 (missing the pro-domain) induces apoptosis in MCF7 breast carcinoma cells. Importantly, upon receipt of a death stimulus, endogenous ICE-LAP3 is processed to its subunit forms, suggesting a physiological role in cell death. This is the first report to demonstrate processing of a native ICE/*ced-3* family member during execution of the death program and the first description of the subcellular localization of an ICE/*ced-3* family member.

Apoptosis, or programmed cell death, is essential for the development and homeostasis of multicellular organisms (1). It is an active form of cellular suicide encoded by an endogenous program that can be triggered by either internal or external cues. The morphological alterations of programmed cell death include cellular shrinkage, membrane blebbing, and chromatin condensation (2). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, acquired immunodeficiency syndrome, and neurodegenerative disorders (3-5).

Despite its biological importance, the molecular mechanism behind apoptosis remains to be defined. Recently, systematic genetic analysis of *Caenorhabditis elegans* has identified three genes (*ced-3*, *ced-4*, and *ced-9*) that are important in the regulation of nematode cell death. The proteins encoded by *ced-3*

and *ced-4* are required for all somatic cell deaths that occur during the development of *C. elegans* (6). Mutations of *ced-3* and *ced-4* abolish the apoptotic capability of cells that normally die during development (7). By contrast, *ced-9*, which encodes a protein that is homologous to the mammalian gene *bcl-2*, functions to suppress somatic cell death in the nematode (8). The function of *ced-9* can be partially substituted by ectopic expression of *bcl-2* (9). These results suggest that components of the apoptotic machinery are conserved throughout evolution and that mammalian counterparts of *ced-3* and *ced-4* may play an important role in the mammalian cell death pathway. To date, no homologues of *ced-4* have been identified. By contrast, numerous relatives of *ced-3* have been characterized, comprising a new gene family of cysteine proteases.

The first mammalian homologue of *ced-3* identified was interleukin-1 β -converting enzyme (ICE)¹ (10), a cysteine protease involved in the processing and activation of pro-interleukin-1 β to an active cytokine (11, 12). Overexpression of ICE or Ced-3 in Rat-1 cells induced apoptosis, suggesting that ICE may act as the functional mammalian homologue of Ced-3 (13). However, later studies refute this possibility, since ICE-deficient mice develop normally and express no overt defects in apoptosis, except possibly in the Fas pathway (14, 15). Furthermore, apoptotic extracts prepared from chicken DU249 cells failed to cleave the primary substrate of ICE, pro-interleukin-1 β (16). Instead, an ICE-like activity in these extracts, termed pICE, cleaved the nuclear enzyme poly(ADP-ribose) polymerase (PARP) into characteristic fragments (16). Purified ICE failed to cleave PARP (16, 17), suggesting that pICE was distinct from ICE. These observations, along with others, suggest that an ICE-like enzyme, rather than ICE itself, plays a role in the mammalian cell death pathway.

Five members of the ICE/Ced-3 family have been recently identified and include Nedd-2/ICH1 (18, 19), Yama/CPP32/Apopain (17, 20, 21), TX/ICH2/ICE rel-II (22-24), ICE rel-III (22), and Mch2 (25). All family members share sequence homology with ICE/Ced-3 and contain an active site QACRG pentapeptide in which the cysteine residue is catalytic. Overexpression of these proteins in a variety of cells causes apoptosis.

Among the ICE/Ced-3 family members thus far cloned, evidence is growing that Yama may act as a distal effector of the apoptotic machinery. Yama has been shown to cleave the death substrate PARP, in addition to being inhibitable by the cowpox serpin, CrmA (17). Activated Yama (or Apopain), comprised of p17 and p12 subunits, was purified from cell extracts using a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U39613.

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¹ The abbreviations used are: ICE, interleukin-1 β -converting enzyme; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; TNF, tumor necrosis factor; PBS, phosphate-buffered saline.

tetrapeptide aldehyde inhibitor corresponding to amino acids at the PARP cleavage site (21). Depletion of Yama from these extracts abrogated their apoptotic potential *in vitro* (21). This apoptotic activity could be restored by adding back purified Yama to the depleted extracts (21). Though the evidence is compelling that Yama serves as a functional mammalian homologue of Ced-3, one cannot exclude a role for other ICE/Ced-3 family members in the cell death pathway. For instance, Yama may be the distal effector of a proteolytic cascade that is comprised of (or activated by) other related family members. A precedent for this is the activation of Yama by purified ICE or granzyme B *in vitro* (17, 26). Alternatively, there may exist a redundant cell death pathway in which individual ICE/Ced-3 family members play a role.

Here we report the cloning and characterization of a novel member of the ICE/Ced-3 family designated ICE-LAP3 (for ICE-Like Apoptotic Protease 3) that is closely related to the death effector Yama. ICE-LAP3 is expressed in a variety of tissues and cell lines. Overexpression of ICE-LAP3 in MCF7 cells induces cell death, and mutation of the putative catalytic cysteine residue abolishes its apoptotic potential. Stimulation of the cytokine death receptors, Fas/APO-1 or TNFR-1, triggers processing of pro-ICE-LAP3 to active p20 and p12 subunits. Taken together, our results suggest that ICE-LAP3 is likely a cysteine protease that may have a role in cytokine-mediated cell death.

MATERIALS AND METHODS

Cloning of Human ICE-LAP3—The cDNA corresponding to ICE-LAP3 was identified as a sequence homologous to ICE on searching the Human Genome Sciences private data base using established EST methods (27, 28).

DNA Sequencing—The sequence of ICE-LAP3 was confirmed by sequencing plasmid DNA template on both strands by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.). Sequence alignments were performed using DNASTAR Megalign software.

Expression Constructs—The p28 version of ICE-LAP3, which lacks the first 53 N-terminal amino acids that encode the putative prodomain was generated by PCR and subcloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). The upstream PCR primer (CGGGGTACCGCCATGCGAGTGCCTACATATCAGTAC) encoded an artificial initiator methionine within Kozak's consensus (italics) and a custom *KpnI* restriction site (underlined). The downstream primer (GCTCTAGATTAGATGTAGCGGTATGTCTTGAC-TGAAGTAGAGTTCC) encoded an AU1 epitope tag (DTYRYI; italics) and an in-frame stop codon (underlined). Alteration of the active site cysteine 184 to alanine was accomplished by site-directed mutagenesis employing a four-primer two-step PCR protocol as described previously (29). Sequences of the mutagenic oligonucleotides were as follows with the altered codon underlined: CAAACTTCTTCTTCATTTCAGGCTGCTC-GAGGGACCGAGCTTGATGA and TCATCAAGCTCGGTCCCTCGAG-CAGCCTGAATGAAGAAGAGTTTG. The presence of the introduced mutation and fidelity of PCR replication were confirmed by sequence analysis.

Northern Blot Analysis—Adult and fetal human multiple tissue Northern blots (Clontech, Palo Alto, CA) were hybridized, according to the manufacturer's instructions, using a radiolabeled cDNA insert from a *KpnI/XbaI* digestion of pcDNA3-ICE-LAP3.

Cell Lines, TNF, and Anti-Fas Antibody—MCF7 cells, BJAB cells, and derived vector, CrmA and CrmA mutant stable transfectants were described previously (17, 30). Jurkat cells were maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine, penicillin/streptomycin, and nonessential amino acids. Recombinant TNF (specific activity, 6.27×10^7 units/mg) was purchased from Genentech (South San Francisco, CA). Anti-Fas (anti-APO-1) antibody was kindly provided by Dr. P. H. Kramer (31).

Preparation of Cells for Analysis of ICE-LAP3 Processing—Jurkat cells, BJAB cells, and derived BJAB transfectants were aliquoted at a concentration of 5×10^6 /ml into T50 tissue culture flasks (Falcon), with 10 ml of medium in each flask. The following day, cells were treated with anti-Fas antibody (100 ng/ml) plus protein A (10 μ g/ml) (Sigma) for the indicated time periods, and cells were harvested by centrifugation

and washed once with PBS and lysed in 1 ml of PBS-TDS lysis buffer (0.38 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS) plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml aprotinin, 0.5 mg/ml antipain, and 0.5 mg/ml pepstatin).

MCF7 cells and derived transfectants were plated in 150-mm dishes and grown to 80% confluency. Cells were treated with TNF (40 ng/ml) for 22 h, harvested by scraping, recovered by centrifugation, washed once with PBS, and lysed as above.

Immunoprecipitation and Western Blot Analysis—Polyclonal anti-ICE-LAP3 antibody was generated by immunizing rabbits with the synthetic peptide KPDRSSFVPSLFSKKKKK, corresponding to the N-terminal portion of the putative p20 subunit of ICE-LAP3 and conjugated to diphtheria toxoid (Chiron, San Diego, CA). Immunoprecipitation and Western analysis were performed as described previously (32). The anti-ICE-LAP3 antiserum was used at a dilution of 1:100 for immunoprecipitation and 1:1000 for Western analysis. The secondary antibody, an anti-rabbit immunoglobulin labeled with horseradish peroxidase, was used at a dilution of 1:10,000. Visualization of the signal was by ECL (Amersham Corp.).

Immunostaining—293T cells were grown to 80% confluency on gelatin-coated glass coverslips while 1×10^6 Jurkat cells were cytocentrifuged onto glass slides. Cells were fixed and permeabilized with 100% methanol at -20°C for 10 min. The cells were subsequently washed with PBS and incubated with either a 1:100 dilution of preimmune serum, anti-LAP3 antiserum, or peptide-blocked anti-LAP3 antiserum. After the PBS wash, cells were incubated with 1:1000 fluorescein isothiocyanate anti-rabbit antibody (Sigma) and subsequently washed with PBS and visualized by fluorescence microscopy (33).

Cell Death Assay—MCF7 cells were transiently transfected as described previously (33). Briefly, 2.5×10^6 MCF7 cells were transfected with 0.25 μ g of the reporter plasmid pCMV β -galactosidase plus 1.5 μ g of test plasmid in 6-well tissue culture dishes using Lipofectamine as per the manufacturer's instructions. The transfection was carried out in 1 ml of Opti-MEM minimal medium (Life Technologies, Inc.), and after 5 h, 1 ml of serum-containing growth medium was added. Two days later, the cells were fixed with 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β -D-galactoside for 4 h. Cells were visualized by phase-contrast microscopy. At least 300 β -galactosidase-positive cells were counted for each transfection ($n = 3$) and identified as apoptotic or nonapoptotic based on morphological alterations typical of adherent cells undergoing apoptosis including becoming rounded, condensed, and detaching from the dish (2).

RESULTS AND DISCUSSION

Cloning of ICE-LAP3—The Human Genome Sciences human cDNA data base was searched for genes related to ICE/Ced-3. Six cDNAs related to the ICE/Ced-3 gene family were isolated, and all except one were previously described (10, 17, 19–25). In this report, we characterize a 2.4-kilobase cDNA that contains an open reading frame beginning with an initiator methionine (34) and ending 912 nucleotides later at an Opal codon. Given the presence of an in-frame stop codon 285 base pairs upstream of the initiator methionine and the size of the transcript (2.4 kilobases; see Fig. 2A), it is likely that the full-length coding sequence was identified. This gene encodes a previously undescribed protein of 304 amino acids with a predicted molecular mass of 35 kDa, designated ICE-LAP3 (Fig. 1A).

ICE-LAP3 Is a Novel Member of the ICE/Ced-3 Gene Family—A BLAST search of the GenBank protein data base revealed that the predicted protein sequence of ICE-LAP3 is similar to the *C. elegans* Ced-3 protein and classifies ICE-LAP3 as a novel member of the ICE/Ced-3 gene family. Of the family members thus far cloned, ICE-LAP3 is the most closely related homologue to the mammalian cell death effector, Yama/CPP32/Apopain (17, 20, 21). ICE-LAP3 has 58% sequence identity (75% similarity) with Yama, 40% identity (57% similarity) with Mch2a, and less than 35% identity (55% similarity) with the other ICE/Ced-3 family members. Like Yama, ICE-LAP3 is highly homologous to Ced-3, sharing 35% sequence identity (54% similarity).

Yama is an Asp-specific cysteine protease responsible for the cleavage of the death substrate PARP (17, 21). The full-length-

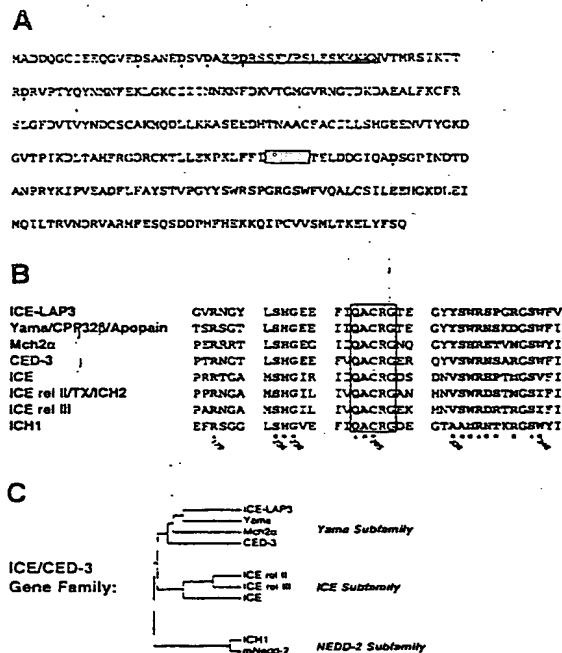


FIG. 1. Sequence analysis of ICE-LAP3. **A**, deduced amino acid sequence of ICE-LAP3. The conserved pentapeptide QACRG is boxed. Putative Asp cleavage sites are indicated with an asterisk. The underlined sequence corresponds to the synthetic peptide used to generate anti-ICE-LAP3 antibody. **B**, sequence alignment of known members of the human ICE/Ced-3 family and the nematode gene *ced-3*. The pentapeptide QACRG is boxed. Based on the x-ray crystal structure of ICE, the residues involved in catalysis and substrate binding are shown in color, and the numbers correspond to the position of residues within ICE. Red-filled circles are catalytic; green-filled triangles represent the binding pocket for the carboxylate of the P₁ Asp; blue-filled squares indicate the residues adjacent to the P₂-P₄ amino acids. **C**, phylogenetic analysis of the ICE/*ced-3* gene family.

p32 form, or Pro-Yama, requires proteolytic processing to active p17 and p12 subunits (21). The majority of sequence homology between Yama and ICE-LAP3 lies within a region of ICE-LAP3 that corresponds to the active subunits of Yama. Comparison with Yama would suggest that ICE-LAP3 contains an N-terminal aspartate cleavage site and an internal cleavage site defining two subunits that are not separated by a linker peptide (Fig. 1A). The QACRG pentapeptide, conserved in all family members, is likewise conserved in ICE-LAP3. In addition, based on the x-ray crystal structure of ICE (35, 36), the amino acid residues His²³⁷, Gly²³⁸, and Cys²⁸⁵ of ICE are involved in catalysis, while the residues Arg¹⁷⁹, Gln²³³, and Arg³⁴¹ form a binding pocket for the carboxylate side chain of the P₁ aspartic acid. These six amino acids are conserved in all ICE/Ced-3 family members including ICE-LAP3 (Fig. 1B). However, residues that form the P₂-P₄ binding pockets are not widely conserved among family members, suggesting that they may determine substrate specificity.

Phylogenetic analysis of the ICE/*ced-3* gene family revealed three subfamilies (Fig. 1C). Yama/CPP32/Apopain, ICE-LAP3, and Mch2 are closely related to *C. elegans* Ced-3 and comprise the Yama subfamily. ICE and the ICE-related genes, TX/ICE rel I/CH2 and ICE rel II, form the ICE subfamily, while ICH1 and its mouse homologue, Nedd-2, form the Nedd-2 subfamily.

Distribution of ICE-LAP3—Northern blot analysis revealed that ICE-LAP3 is constitutively expressed in a variety of fetal and adult human tissues with small amounts in the fetal brain (Fig. 2A). The mRNA transcript is approximately 2.4 kilobases,

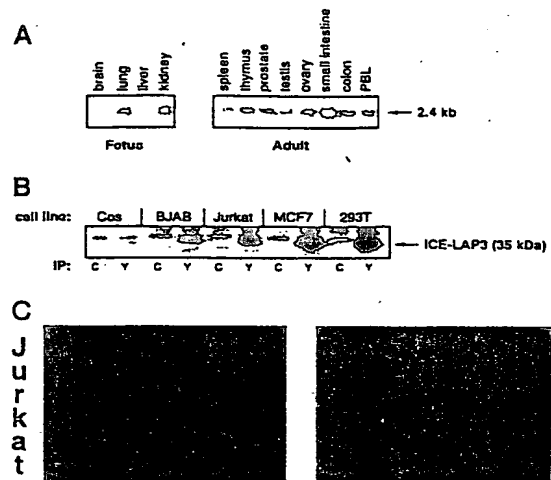


FIG. 2. ICE-LAP3 expression and subcellular localization. **A**, a human adult and fetal tissue poly(A)⁺ Northern blot (Clontech) was probed with ³²P-labeled ICE-LAP3 cDNA. PBL, peripheral blood leukocyte. **B**, a variety of human cell lines were examined for expression of endogenous ICE-LAP3 protein. Cell lysates were immunoprecipitated (IP) with anti-ICE-LAP3 antibody (Y) or control pre-immune serum (C) and immunoblotted with anti-ICE-LAP3 antibody. **C**, immunolocalization of ICE-LAP3 using the anti-LAP3 polyclonal antisera. Anti-LAP3 reactivity was visualized with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. Jurkat T cells were stained with peptide-blocked anti-LAP3 serum (left) and anti-LAP3 serum (right).

consistent with the size of the cDNA clones isolated. Using a rabbit anti-peptide antibody directed against ICE-LAP3, a variety of cell lines were examined for expression of ICE-LAP3 protein, which was detected to a variable extent in all cell lines analyzed (Fig. 2B). Endogenous ICE-LAP3 migrated at approximately 35 kDa, consistent with the predicted molecular mass.

Cellular Localization of ICE-LAP3—Intracellular localization of endogenous ICE-LAP3 was determined using the above described anti-LAP3 antibody. In Jurkat T cells, ICE-LAP3 localized diffusely to the cytoplasm and juxtamembrane structures (Fig. 2C). Similar results were obtained using 293T cells (data not shown). The specificity of this staining was confirmed using preimmune serum and peptide-blocked anti-LAP3 serum. This is the first reported immunolocalization of an endogenous ICE/Ced-3 family member and is consistent with previous reports suggesting that the death effector machinery resides within the cytoplasm and not in the nucleus (37).

Overexpression of ICE-LAP3 in MCF7 Cells Induces Apoptosis—All ICE/Ced-3 family members have been shown to induce apoptosis when overexpressed in various cell lines. To determine whether ICE-LAP3 may have a role in cell death, an expression construct encoding the full-length protein was transfected into MCF7 breast carcinoma cells and subsequently assessed for apoptotic features. Unlike full-length ICE, expression of full-length ICE-LAP3 failed to induce apoptosis (data not shown). However, expression of a truncated derivative of ICE-LAP3 (pcDNA3 AU1-ICE-LAP3 p28), which lacked 53 N-terminal amino acids corresponding to the putative prodomain, caused cell death (Fig. 3A). The ICE-LAP3 p28-transfected cells displayed morphological alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (Fig. 3B). The nuclei of the rounded MCF7 cells exhibited apoptotic morphology as assessed by propidium iodide staining (data not shown). To determine whether amino acid residue Cys¹⁸⁶, corresponding to the catalytic Cys²⁸⁵ of ICE, was essential for apoptotic activity, a mutant of ICE-LAP3 was generated in which the cysteine

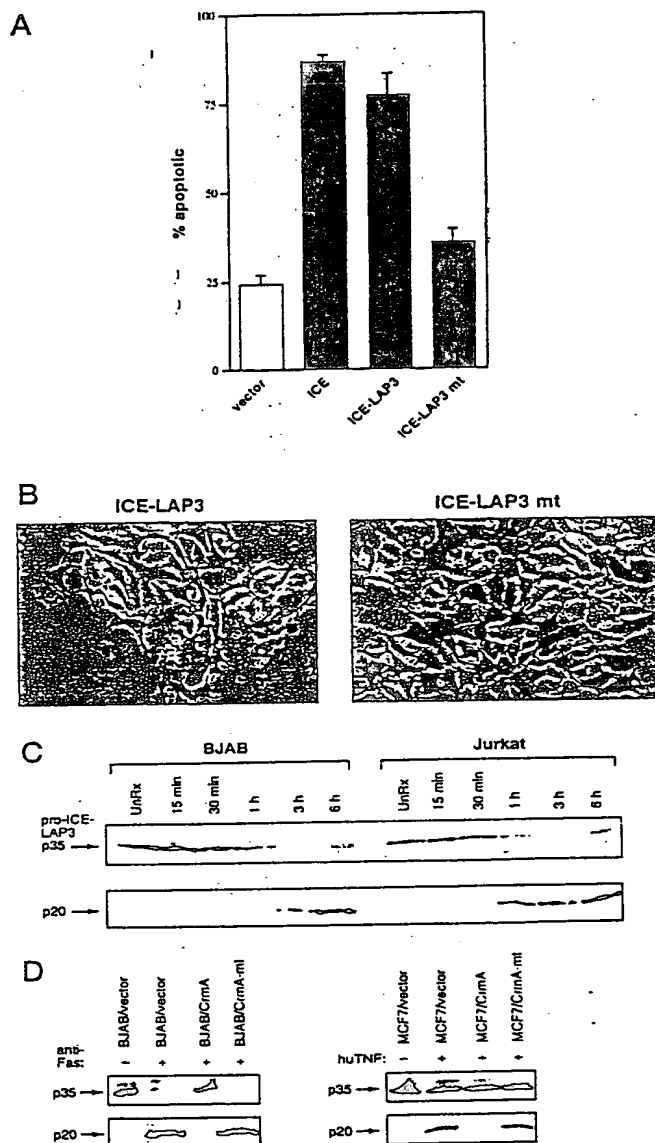


FIG. 3. ICE-LAP3 induces apoptosis and is activated during Fas- and TNF-induced cell death. **A**, MCF7 cells were transiently transfected with the reporter gene β -galactosidase and either the truncated form of ICE-LAP3 (p28) or the mutant version with the catalytic cysteine residue inactivated (ICE-LAP3 mutant), ICE, or a vector control as described under "Materials and Methods." Percent apoptotic cells represents the mean value from three independent experiments (mean \pm S.D.). **B**, transfected cells were stained with 5-bromo-4-chloro-3-indolyl β -D-galactoside and examined by phase contrast microscopy. **C**, a time course of pro-ICE-LAP3 proteolytic processing generating an active p20 subunit during Fas-induced apoptosis of BJAB and Jurkat cells. 5×10^6 BJAB or Jurkat cells were treated with anti-Fas antibody (100 ng/ml) plus protein A (10 μ g/ml) for the indicated time periods, and cell lysates were analyzed by immunoprecipitation and Western blot analysis with anti-ICE-LAP3 antiserum. **D**, activation of ICE-LAP3 during anti-Fas and TNF treatment is blocked by CrmA. Cells were treated with anti-Fas antibody or TNF and prepared as in **C**. **Left panel**, 5×10^6 BJAB cells or derived transfectants were treated with anti-Fas antibody (100 ng/ml) plus protein A (10 μ g/ml) for 6 h. **Right panel**, MCF7 or derived transfectants were treated with TNF (40 ng/ml) for 22 h.

residue was altered to an alanine. Overexpression of the mutant form of ICE-LAP3 did not induce apoptotic changes in MCF7 cells (Fig. 3, A and B). Taken together, these results suggest that the activity of ICE-LAP3 is tightly regulated in mammalian cells and that activation of ICE-LAP3 likely requires removal of the pro-domain. This is consistent with the finding that overexpression of the pro-form of Yama (Pro-Yama) in MCF7 cells also failed to induce cell death (data not shown).

ICE-LAP3 Is Activated during Fas- and TNF-induced Apoptosis—Two cell surface cytokine receptors, Fas/APO-1 and the p55 receptor for tumor necrosis factor (TNFR-1), have been shown to trigger apoptosis by their respective natural ligands or specific agonist antibodies (30). To determine whether ICE-LAP3 may be a component of the Fas- and TNF-induced cell death pathways, concomitant processing of endogenous pro-ICE-LAP3 was assessed. Over a time course of incubation with anti-Fas antibody, pro-ICE-LAP3 was processed to form p20 and p12 subunits in both Jurkat T cells and BJAB B cells (the anti-LAP3 antibody generated was directed against the p20 subunit, which could therefore be detected) (Fig. 3C). Similar results were observed during TNF-induced cell death of MCF7 cells (Fig. 3D). CrmA, a poxvirus gene product, has been shown to potentially block both Fas- and TNF-induced cell death (30). Engagement of Fas and TNFR in CrmA-expressing cells abrogated ICE-LAP3 activation as well as cell death (Fig. 3D) (30). As expected, ICE-LAP3 was processed in inactive mutant CrmA (17) expressing cell lines (Fig. 3D).

In conclusion, we have identified a novel death-inducing protein of the ICE/Ced-3 family. ICE-LAP3 was immunolocalized to the cytoplasm, where the death effector machinery is thought to reside (37). This is the first report describing the activation of an endogenous ICE/Ced-3 cysteine protease during Fas- and TNF-induced cell death, suggesting that it is a mediator of the cytokine-mediated cell death pathway. Further, there is compelling evidence that a family member, Yama, acts as a distal effector of the cell death pathway (17, 21). The high homology between Yama and ICE-LAP3 is intriguing and raises many important questions about a potential role of ICE-LAP3 in a general cell death program. One possibility is that ICE-LAP3 also acts as an effector in a redundant cell death pathway distinct from the one utilizing Yama. Alternatively, ICE-LAP3 could act as an upstream enzyme, or "Yama convertase," responsible for the processing of Pro-Yama to an active death protease. The cloning and characterization of the Yama-related protein ICE-LAP3 will help address these specific questions and, in general, enhance our understanding of the cell death machinery and the proteases that compose it.

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Exhibit D

	10	20	30	40	
1	A T G G C A G A T G A T T C A G G G C T G T A T T G A A G A G C A G G G G G T T				PF140
1	A T G G C A G A T G A T - C A G G G C T G T A T T G A A G A G C A G G G G G T T				Duan
	50	60	70	80	
41	G A G G A T T C A G C A A A T G A A G A T T C A G T G G A T G C T A A G C C A G				PF140
40	G A G G A T T C A G C A A A T G A A G A T T C A G T G G A T G C T A A G C C A G				Duan
	90	100	110	120	
81	A C C G G T C C T C G T T T G T A C C G T C C C T C T T C A G T A A G A A G A A				PF140
80	A C C G G T C C T C G T T T G T A C C G T C C C T C T T C A G T A A G A A G A A				Duan
	130	140	150	160	
121	G A A A A A T G T C A C C A T G C G A T C C A T C A A G A C C A C C C G G G A C				PF140
120	G A A A A A T G T C A C C A T G C G A T C C A T C A A G A C C A C C C G G G A C				Duan
	170	180	190	200	
161	C G A G T G C C T A C A T A T C A G T A C A A C A T G A A T T T T G A A A A G C				PF140
160	C G A G T G C C T A C A T A T C A G T A C A A C A T G A A T T T T G A A A A G C				Duan
	210	220	230	240	
201	T G G G C A A A T G C A T C A T A A T A A A C A A C A A G A A C T T T G A T A A				PF140
200	T G G G C A A A T G C A T C A T A A T A A A C A A C A A G A A C T T T G A T A A				Duan
	250	260	270	280	
241	A G T G A C A G G T A T G G G C G T T C G A A A C G G A A C A G A C A A A G A T				PF140
240	A G T G A C A G G T A T G G G C G T T C G A A A C G G A A C A G A C A A A G A T				Duan
	290	300	310	320	
281	G C C G A G G C G C T C T T C A A G T G C T T C C G A A G C C T G G G T T T T G				PF140
280	G C C G A G G C G C T C T T C A A G T G C T T C C G A A G C C T G G G T T T T G				Duan
	330	340	350	360	
321	A C G T G A T T G T C T A T A A T G A C T G C T C T T G T G C C A A G A T G C A				PF140
320	A C G T G A T T G T C T A T A A T G A C T G C T C T T G T G C C A A G A T G C A				Duan
	370	380	390	400	
361	A G A T C T G C T T A A A A A A G C T T C T G A A G A G G A C C A T A C A A A T				PF140
360	A G A T C T G C T T A A A A A A G C T T C T G A A G A G G A C C A T A C A A A T				Duan
	410	420	430	440	
401	G C C G C C T G C T T C G C C T G C A T C C T C T T A A G C C A T G G A G A A G				PF140
400	G C C G C C T G C T T C G C C T G C A T C C T C T T A A G C C A T G G A G A A G				Duan

	450	460	470	480	
441	A A A A T G T A A T T T A T G G G A A A G A T G G T G T C A C A C C A A T A A A				PF140
440	A A A A T G T A A T T T A T G G G A A A G A T G G T G T C A C A C C A A T A A A				Duan
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	490	500	510	520	
481	G G A T T T G A C A G C C C A C T T T A G G G G G G A T A G A T G C A A A A C C				PF140
480	G G A T T T G A C A G C C C A C T T T A G G G G G G A T A G A T G C A A A A C C				Duan
<hr/>					
	530	540	550	560	
521	C T T T T A G A G A A A C C C A A A C T C T T C T T C A T T C A G G C T T G C C				PF140
520	C T T T T A G A G A A A C C C A A A C T C T T C T T C A T T C A G G C T T G C C				Duan
<hr/>					
	570	580	590	600	
561	G A G G G A C C G A G C T T G A T G A T G G C A T C C A G G C C G A C T C G G G				PF140
560	G A G G G A C C G A G C T T G A T G A T G C C A T C C A G G C C G A C T C G G G				Duan
<hr/>					
	610	620	630	640	
601	G C C C A T C A A T G A C A C A G A T G C T A A T C C T C G A T A C A A G A T C				PF140
600	G C C C A T C A A T G A C A C A G A T G C T A A T C C T C G A T A C A A G A T C				Duan
<hr/>					
	650	660	670	680	
641	C C A G T G G A A G C T G A C T T C C T C T T C G C C T A T T C C A C G G T T C				PF140
640	C C A G T G G A A G C T G A C T T C C T C T T C G C C T A T T C C A C G G T T C				Duan
<hr/>					
	690	700	710	720	
681	C A G G C T A T T A C T C G T G G A G G A G C C C A G G A A G A G G C T C C T G				PF140
680	C A G G C T A T T A C T C G T G G A G G A G C C C A G G A A G A G G C T C C T G				Duan
<hr/>					
	730	740	750	760	
721	G T T T G T G C A A G C C C T C T G C T C C A T C C T G G A G G A G C A C G G A				PF140
720	G T T T G T G C A A G C C C T C T G C T C C A T C C T G G A G G A G C A C G G A				Duan
<hr/>					
	770	780	790	800	
761	A A A G A C C T G G A A A T C A T G C A A A T C C T C C A C C A G G G T G A A T				PF140
760	A A A G A C C T G G A A A T C A T G C A G A T C C T C - A C C A G G G T G A A T				Duan
<hr/>					
	810	820	830	840	
801	G A C A G A G T T G C C A G G C A C T T T G A G T C T C A G T C T G A T G A C C				PF140
799	G A C A G A G T T G C C A G G C A C T T T G A G T C T C A G T C T G A T G A C C				Duan
<hr/>					
	850	860	870	880	
841	C A C A C T T C C A T G A G A A G A A G C A G A T C C C C T G T G T G G T C T C				PF140
839	C A C A C T T C C A T G A G A A G A A G C A G A T C C C C T G T G T G G T C T C				Duan

	890	900	910
881	CATGCTCACCAAGGAAGTCTACTTCAGTCAATAG		
879	CATGCTCACCAAGGAAGTCTACTTCAGTCAATAG		

PF140
Duan

Decoration 'Decoration #1': Shade (with solid black) residues that match PF140 exactly.

Exhibit E

	10	20	30	40	
1	M R A G T R V A L G S S T P A E R T T P S Q G R C K P R P A L P R A A P P S A A				PF140.aa
1	M A D D - - - - -				Duan.aa
	50	60	70	80	
41	P R L L V S L S L K G P Q T L A A E R R R E T V P V P A A L P P W E R W Q M I Q				PF140.aa
5	- - - - -				Duan.aa
	90	100	110	120	
81	G C I E E Q G V E D S A N E D S V D A K P D R S S F V P S L F S K K K K N V T M				PF140.aa
6	G C I E E Q G V E D S A N E D S V D A K P D R S S F V P S L F S K K K K N V T M				Duan.aa
	130	140	150	160	
121	R S I K T T R D R V P T Y Q Y N M N F E K L G K C I I I N N K N F D K V T G M G				PF140.aa
46	R S I K T T R D R V P T Y Q Y N M N F E K L G K C I I I N N K N F D K V T G M G				Duan.aa
	170	180	190	200	
161	V R N G T D K D A E A L F K C F R S L G F D V I V Y N D C S C A K M Q D L L K K				PF140.aa
86	V R N G T D K D A E A L F K C F R S L G F D V I V Y N D C S C A K M Q D L L K K				Duan.aa
	210	220	230	240	
201	A S E E D H T N A A C F A C I L L S H G E E N V I Y G K D G V T P I K D L T A H				PF140.aa
126	A S E E D H T N A A C F A C I L L S H G E E N V I Y G K D G V T P I K D L T A H				Duan.aa
	250	260	270	280	
241	F R G D R C K T L L E K P K L F F I Q A C R G T E L D D G I Q A D S G P I N D T				PF140.aa
166	F R G D R C K T L L E K P K L F F I Q A C R G T E L D D A I Q A D S G P I N D T				Duan.aa
	290	300	310	320	
281	D A N P R Y K I P V E A D F L F A Y S T V P G Y Y S W R S P G R G S W F V Q A L				PF140.aa
206	D A N P R Y K I P V E A D F L F A Y S T V P G Y Y S W R S P G R G S W F V Q A L				Duan.aa
	330	340	350	360	
321	C S I L E E H G K D L E I M Q I L H Q G E .				PF140.aa
246	C S I L E E H G K D L E I M Q I L T R V N D R V A R H F E S Q S D D P H F H E K				Duan.aa
	370				
342					PF140.aa
286	K Q I P C V V S M L T K E L Y F S Q				Duan.aa

Decoration 'Decoration #1': Shade (with solid black) residues that match the Consensus exactly.